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TITLE: Functional Proteomics to Identify Moderators of CD8+ T Cell Function in Melanoma

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14. ABSTRACT In the funding period we have optimized phage-screens to select clones that differentially bind to either tumor infiltrating cytotoxic (CD8+) lymphocytes, activated CD8+ lymphocytes from the spleen, or un-activated naïve CD8+ T cells. We have developed a high-throughput flow cytometric approach that allows us to screen the specificity of several phage clones for each of these CD8+ populations. Using this initial approach we have identified 17 phage that selectively bind TIL rather than effector cells. However, none of these phage influenced CD8+ TIL expansion or function in vitro. Using a novel NextGeneration sequencing approach, we have further defined another 1,000,000 phage that selectively bind TIL, of which 100,000 are unique reads. Highly represented phage have been subcloned and are being tested for in vitro function. We have identified one phage that augments T cell expansion in vitro. Phage have been tested for their ability to image tumors.

15. SUBJECT TERMS Nothing Listed					
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1. Introduction: CD8+ T cells found within tumors are frequently dysfunctional. In many cases, dysfunction is caused by the expression of inhibitory molecules on the T cells that are designed to prevent immunopathology, but in the context of tumors, prevent T cells from performing their effector functions. Studies have shown that blocking these inhibitors with monoclonal antibodies, a process known as checkpoint blockade, can lead to the control of tumor. In melanoma patients, approximately 20% of patients make clinically relevant responses to their tumors in the presence of checkpoint blockade for the inhibitory molecule PD-1. Notably, responsiveness is strongly correlated with the expression of the ligands for PD-1. This result, while promising, begs the question why more patients do not respond. Several other inhibitory molecules have been described, suggesting that restraint of T cell function by inhibitory molecules is a multi-faceted process of critical evolutionary importance, leading to speculation that the full panoply of inhibitory molecules needs to be identified before a significantly greater proportion of patients respond to checkpoint blockade. It also raises the question as to whether different checkpoint blockade molecules are expressed by T cells that infiltrate tumors of different histology, and in different anatomical locations. Contemporary approaches to defining inhibitory molecules have tended to depend upon defining genetic lesions that result in an autoimmune phenotype in mutant mice. Other studies have focused on using gene expression profiling to identify differentially expressed molecules that have homology to identified inhibitory molecules, or contain motifs that have previously been identified to confer inhibitory function. However, this approach suffers from uncertainty about the biology of the identified transcript. To directly identify inhibitory molecules, we have proposed to use phage-display expression libraries to perform functional proteomics to identify molecules expressed on the surface of tumor infiltrating lymphocytes that are absent from resting or effector lymphocytes that are found in non-tumor settings. We then intend to use the phage to image the expression of these molecules in vivo, and determine the functional relevance of the molecules.

2. Keywords: phage-display; CD8+ T cells; checkpoint inhibition; cancer immunotherapy; in vivo imaging.

3. Accomplishments:

A. Major Goals:

1. To identify peptides that selectively bind dysfunctional tumor-infiltrating $CD8^+$ T cells
2. To determine the importance of identified cell surface proteins on $CD8^+$ T cell function
3. To identify the ligands of inhibitory molecules expressed by melanomas

B. Accomplishments:

Phage panning: Using the original approach outlined in the application, we identified a total of 17 unique phage that selectively bind CD8+ TIL but not effector or naïve CD8+ T cells (**Figure 1**). Previous work by our group had shown that phage can selectively bind different myeloid cell subsets; however, this study represented the first attempt to use this process on activated lymphocytes. We made several significant modifications to the technique that allowed more efficient elution of the phage, and developed a serial subtraction process using naïve, memory and effector T cells to remove phage that generally bound T cells, resulting in the first crop of TIL-specific candidates. We found that activated T cells are quite susceptible to the glycine-based elution techniques that are used to isolate phage from bound cells, resulting in very low

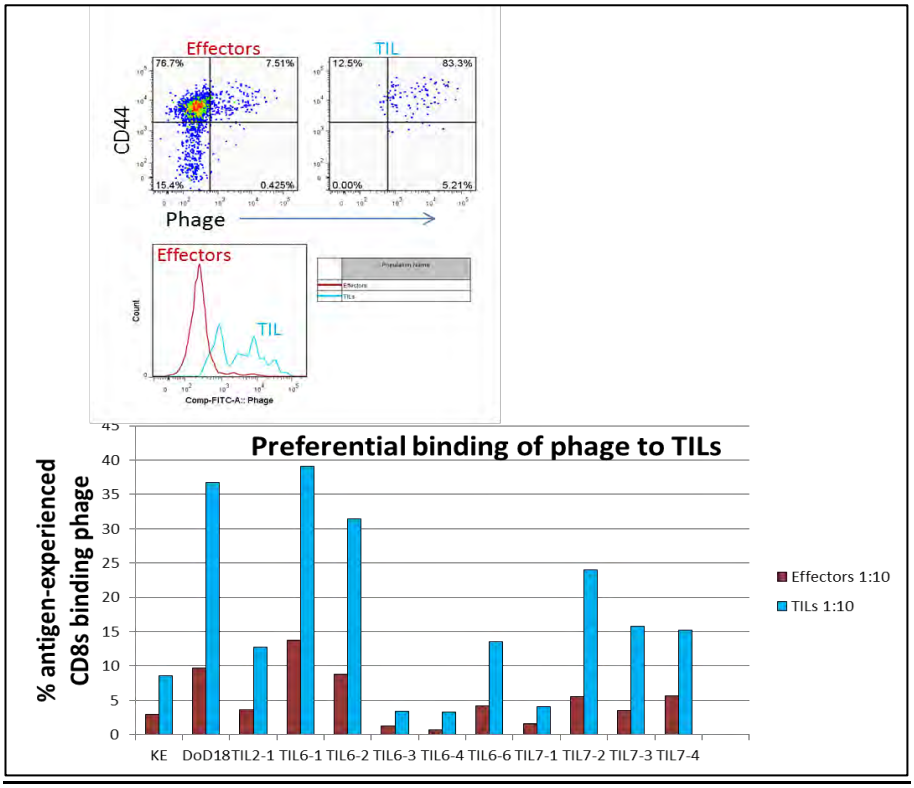


Figure 1. Selective binding of phage to CD8+ TIL in vitro. Plaque purified phage were derived from pools selected on the basis of positive binding to CD8+ TIL and panned against effector and naïve CD8+ T cells. Phage are labelled with fluorochromes and then assessed for binding to T cells excised from either the spleens of mice immunized 7d prior (Effectors, left dot plot) or from d14 CD8+ TIL populations (TIL, right dot plots). Histogram shows relative phage binding. Bar chart shows selective binding of 10 representative phage. KE is a control phage.

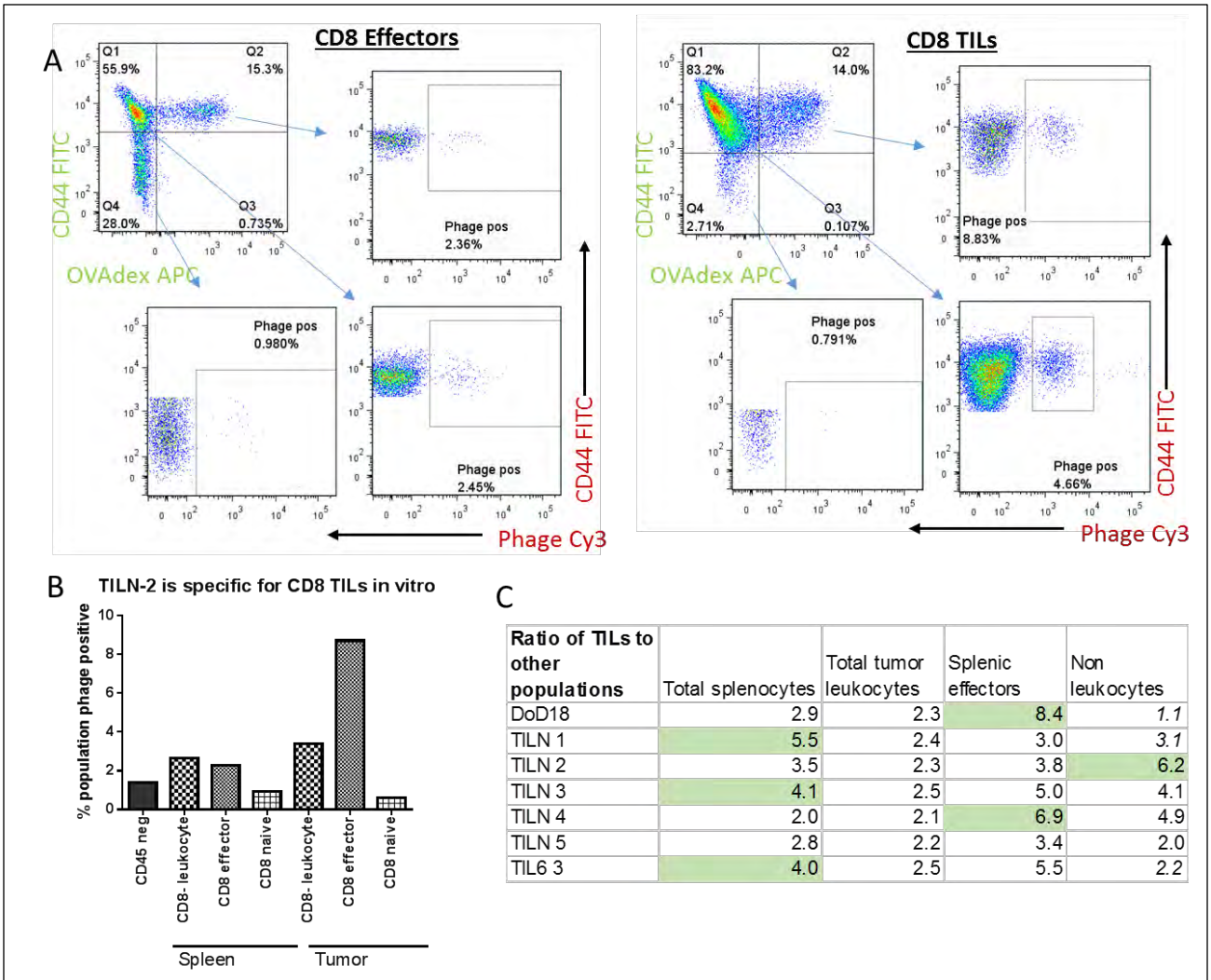
phage yields, possibly due to enmeshment in DNA released from dying cells. We also determined that the antibodies used to isolate the cell populations used for the sequential pans were inhibiting phage binding, and therefore developed an approach to remove them. After optimization, 50 selected phage have been plaque purified, PCR amplified and sequenced across the insertion site. These sequences were compared to a library of known hyper-multiplying sequences to remove phage with growth advantages.

High-throughput screening: To develop flow cytometry-based high throughput screening techniques we assessed two factors. First, we determined whether we could label phage with discrete fluorochromes, allowing us to screen phage in pools. While phage with different flurochromes could be detected when screened individually, when pools of phage were assayed there was a detrimental loss in binding signal intensity, suggesting that the phage could be sterically hindering each other from binding. Therefore we progressed with single phage screens of top candidates. Next, we examined whether the fluorochrome used to label the phage provided any advantage. We found that the label that had been previously used on phage used for in vivo imaging, VT-680, was poorly detected in our cytometers. Screening other labels indicated that while FITC, Pacific Blue and Cy3 labelled phage well, Cy5 labelled phage provided the strongest signal-to-noise ratio, and subsequent phage screens were

performed with Cy5 labelled phage. Finally, we determined the concentration of phage that would provide the optimal signal:noise, and a 1:10 dilution was found to work across many phage screens.

17 unique phage sequences were screened for specific binding by incubating fluorescently-labelled phage with co-cultures of effector T cells (driven by standard immunization techniques) and CD8+ T cells isolated from tumors (**Figure 1**).

In silico phage identification: The process of isolating phage by plaque-purification has proven useful for the proof-of-principle that phage-display approaches can be used to identify molecules that are expressed discretely by TIL compared to other T cells. However, this has proven to be a low-throughput approach that does not sample the pool of selected phage in much depth, and is prone to isolation bias. Therefore we developed a novel approach of using NextGen Sequencing to identify all the phage that bind CD8+ TIL, and then use in silico subtraction techniques to eliminate phage that also bind to other populations of lymphocytes, and cellular subsets screened in other studies. We can now use in silico approaches to identify all the sequences that bind to CD8+ TIL, subtract those that are known to bind to other cell populations, and subclone differentially expressed sequences the phage backbone for functional assessment (or have peptides synthesized). This approach provides two further advantages: we can sequence phage that bind to TIL present in tumors growing in inhibitor molecule knockout mice and thus identify those sequences that selectively bind to those molecules and molecules that have increased expression in the absence of particular inhibitory molecules (i.e. PD-1) that could reflect mechanisms of adaptive resistance; we can use antibodies against known inhibitory molecules to elute phage, thus removing them from the repertoire of molecules. However, this approach has also required us to develop bioinformatics approaches and code that identifies novel sequences and the frequency at which they are expressed in the repertoire of phage binding to TIL or other cellular subsets. Notably, in the first NGS screen we identified many of the phage sequences, or variants thereof, that had been identified by the subtractive-panning approach. As seen in **Figure 2**, NGS-defined phage can bind TIL selectively, but often times only a low percentage of the TIL population bind phage. We are currently unsure whether this means that the molecule is expressed on a small population (which may limit the functional utility of targeting this molecule) or that the phage is binding at low affinity. We will need to identify this molecule in order to differentiate between these possibilities. Interestingly, some phage generally bind hematopoietic cells in tumors but not in spleens (data not shown), and may have general utility for TIL imaging (discussed below) as they bind to a broader population of cells. However, when phage were injected in vivo, we found little evidence of them binding to CD8+ TIL when they were harvested (data not shown). Based on this data, our next steps will be to optimize phage or peptide delivery.



Ratio of TILs to other populations	Total splenocytes	Total tumor leukocytes	Splenic effectors	Non leukocytes
DoD18	2.9	2.3	8.4	*1.1*
TILN 1	5.5	2.4	3.0	*3.1*
TILN 2	3.5	2.3	3.8	6.2
TILN 3	4.1	2.5	5.0	4.1
TILN 4	2.0	2.1	6.9	4.9
TILN 5	2.8	2.2	3.4	2.0
TIL6 3	4.0	2.5	5.5	*2.2*

Figure 2: Selective binding of in silico screened phage to CD8+ TIL. A. Flow cytometry of CD8 effectors (left plots) and CD8+ TIL (right plots) showing the binding of a representative phage to T cells specific for the same antigen at a higher freqeuncy in TIL than effectors. B. Summary of binding of TILN-2 to hematopoietc populations in tumors and spleens of tumor bearing animals. C. Ratio of phage binding to TIL compared to other representative populations of lymphocytes derived from different locations in tumor bearing mice.

Impact of phage on T cell function. As part of the functional proteomics approach taken in this project we hypothesized that select phage could bind to receptors that would either promote CD8+ TIL function by either engaging activating receptors that are absent from the tumor microenvironment, or by blocking inhibitory receptors whose ligands are expressed in the tumor microenvironment. To test this, phage that selectively bound to TIL were co-cultured with TIL either in the presence of activating antibodies to CD3 or the B16cOVA melanoma cell line used in this study, serving as antigen presenting cells. To date, none of the phage that we have screened have promoted the function (proliferation, cytokine production) of CD8+ TIL ex vivo (**Figure 3**). Presumably they bind to proteins that are associated with trafficking or persistence of CD8+ T cells in peripheral tissue rather than secondary lymphoid organs. We could test this

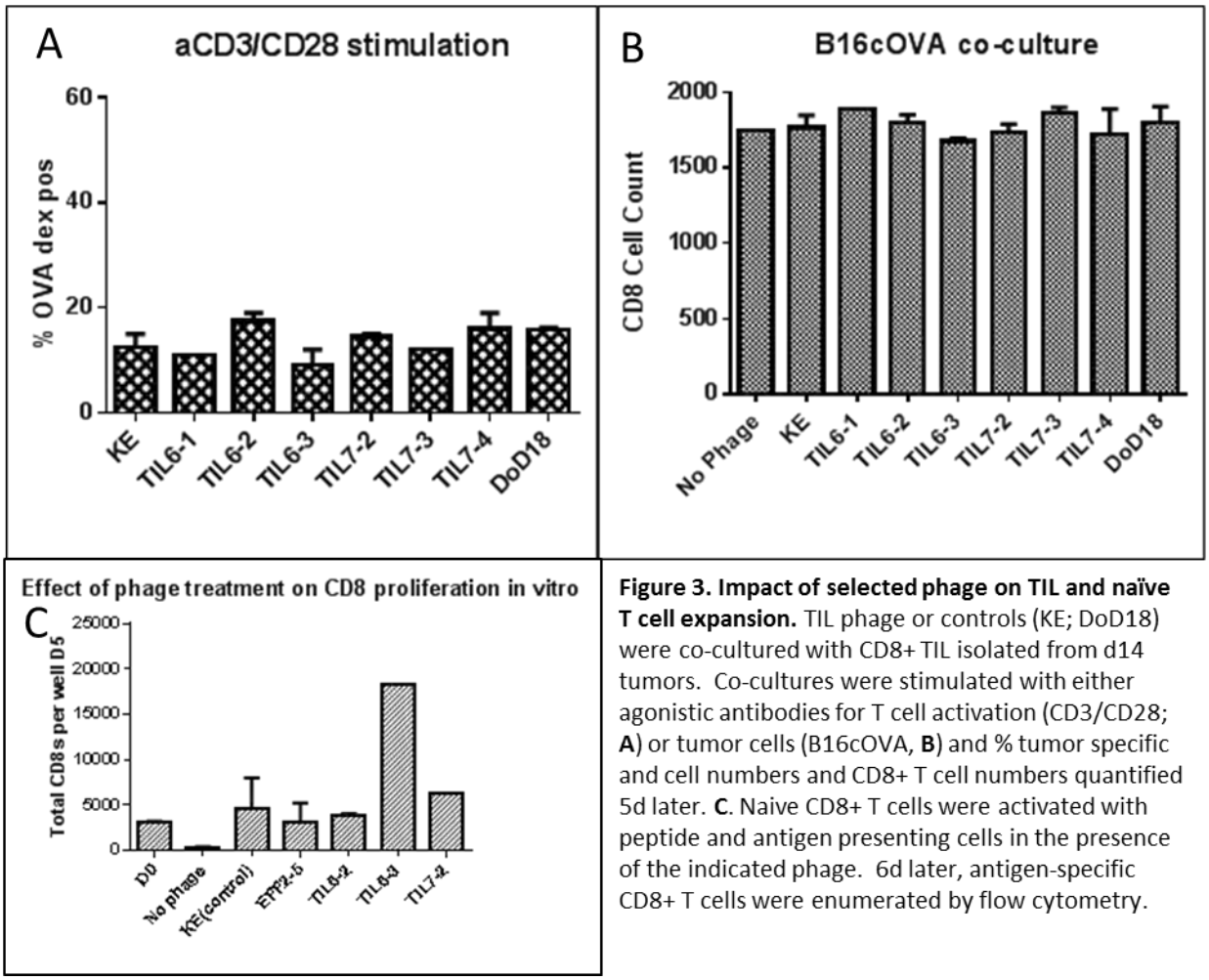


Figure 3. Impact of selected phage on TIL and naïve T cell expansion. TIL phage or controls (KE; DoD18) were co-cultured with CD8+ TIL isolated from d14 tumors. Co-cultures were stimulated with either agonistic antibodies for T cell activation (CD3/CD28; **A**) or tumor cells (B16cOVA, **B**) and % tumor specific and cell numbers and CD8+ T cell numbers quantified 5d later. **C**. Naive CD8+ T cells were activated with peptide and antigen presenting cells in the presence of the indicated phage. 6d later, antigen-specific CD8+ T cells were enumerated by flow cytometry.

possibility by asking whether these phage bind effectors that are found in lung tissue. Alternatively, phage may not sufficiently abrogate receptor-ligand interactions. We could examine this possibility by identifying the ligands of some of the most distinctively binding phage and testing whether antibodies to these ligands influence T cell function. Pertaining to this, we have identified 1 phage that promotes the expansion of control effector CD8+ T cells (**Figure 3**). This suggests that the phage do have the ability to engage molecules on the surface of T cells in manner that is sufficient to manipulate their function, suggesting that further screening of unique phage is warranted. We have sequenced the agonist phage and forwarded it to pull-down studies and mass-spec identification. As none of the phage identified to date have influenced the function of TIL in vitro, we have at this point neither assessed whether any of the phage or their sequences can improve TIL function or tumor control in vivo, nor proceeded to identify the phage binding partners or their ligands. We are confident that our in silico approach is going to identify molecules that are more prevalently expressed on TIL and that some of these will show in vitro activity with respect to promoting TIL function. Once such sequences have been identified, we will determine whether they are commonly expressed by TIL in other cancers, and expressed by human TIL.

Visualization of TIL: A major point of consternation for oncologists and radiologists in the deployment of tumor immunotherapy is that tumors often appear to be growing by radiological criteria (e.g. PET), but this signature can reflect on either true tumor growth or infiltration by

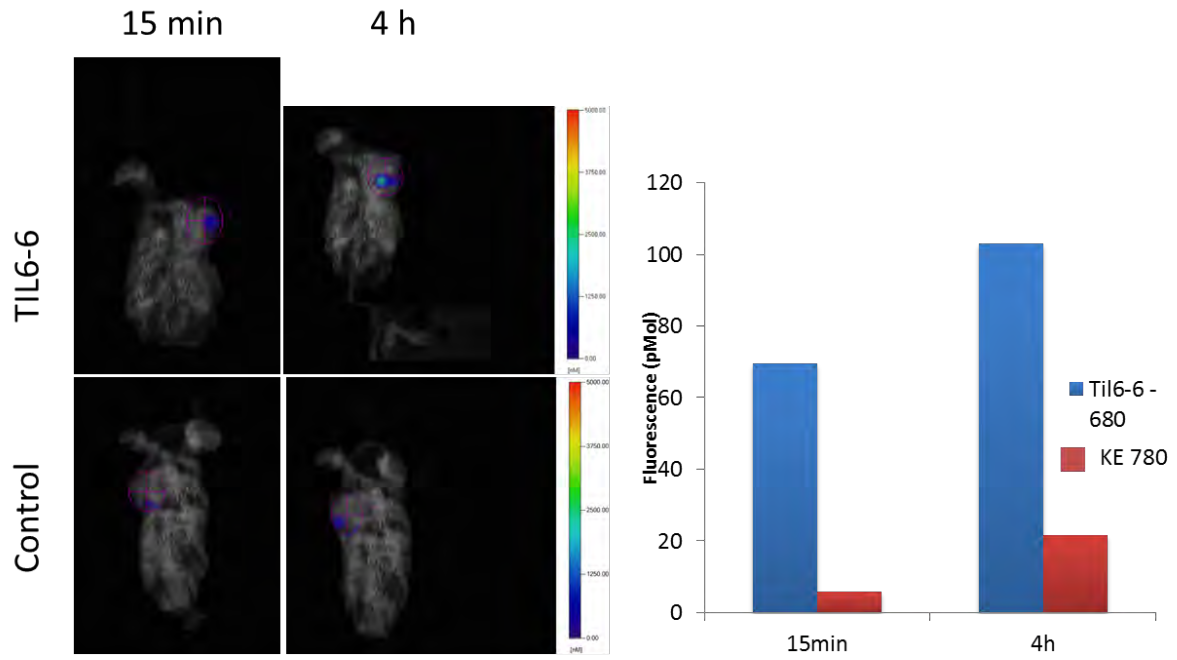


Figure 4: Visualization of tumor by TIL selective phage. Tumors were grown for 12d prior to infusion with 1e9 control (KE) phage and 1e9 TIL6-6. Tumors were imaged by FMT 15 mins and 4h after infusion

metabolically active immune cells. We considered whether phage identified in this study can used to image influx of lymphocytes in tumors. For this purpose, we developed a fluorescence-based tomography (FMT) probe based on the TIL6-6 phage that showed promising selective in vitro binding to CD8+ TIL. Tumor bearing mice were infused with control KE-phage that emit at a discrete wavelength to the TIL6-6 phage, and were imaged at 4h and 20h after transfer. We found evidence that this phage did have increased retention in tumors (**Figure 4**), suggesting selective binding. Interestingly, not all tumors perfused with control phage equivalently, suggesting that phage may have an issue with accessing tumors. We are addressing this by imaging with peptides derived from the phage, and by altering delivery routes for the phage. Further, we found that the greatest proportion of the phage ended in myeloid cells within the tumor, suggesting that the phage are rapidly phagocytosed. These issues are the focus of ongoing efforts to develop the selected sequences for visualization technologies that may play a significant role in discerning patients are responding to immunotherapeutic interventions.

Training and professional development: While training was not an identified goal of this project, Dustin Bauknight (Graduate student) has had opportunities to present these data in research symposia. Andrew Buckner (Lab specialist) has also gained experience in presenting data in the public forum. Both contributors have learned new techniques that broaden their professional experience.

Dissemination of results: To date, dissemination of data has taken place in poster presentations at Departmental research retreats. Mr Bauknight will be presenting our data at an upcoming Biomedical Engineering conference. Dr Bullock has highlighted this collaboration and approach in several research seminars that he has presented at varying universities, and UVA Cancer

Center outreach programs in the community. We anticipate generating multiple manuscripts that report on the construction of the matrices that report on specific cell types that are bound by the phage; the development of the high-throughput approaches for TIL screening; and the imaging capabilities of selected phage sequences.

Future plans: This is the final report.

4. Impact

Principal Disciplines: The results to date indicate that we can use phage-display libraries to identify molecules that are uniquely expressed by T cells that infiltrate tumors. We have integrated this powerful technology with cutting edge research tools (NextGen sequencing; multiparameter single cell flow cytometry) to generate an extensive pool of potentially actionable targets. We present evidence that engaging these molecules has the ability to influence T cell function. We have demonstrated that these molecules can be used to visualize immune cells within tumors. Together, these data indicate that this technique and its subsequent implementation may provide avenues by which we can engender further understanding about T cell function within tumors and identify potential targets for modifying their function. Whether this type of intervention can result in the attenuation of tumor outgrowth remains to be established.

Other Disciplines: Nothing to Report

Technology Transfer: We had extensive discussions with Sanofi about developing the sequences that we identified and the corresponding molecules for commercial purposes. To date, no action has been taken on these discussions.

Society beyond Science and Technology: Nothing to report.

5. Changes/ Problems:

This is the final report. Problems encountered during the project and how they were tackled, or will be in the future development of this project, have been outlined in the Accomplishments section. No further problems are reported.

6. Products

Buckner, A: “Using Phage Display Libraries for Novel Receptor Discovery and *in vivo* Imaging of Tumor Infiltrating Lymphocytes”. UVA Pathology Department Research Retreat; April 2015.

Bauknight, D: "Developing T cell targeted peptides for monitoring immunotherapy response"; UVA BMES student summer seminar series; July 2015

Bauknight, D: "Developing T Cell Targeted Peptides for Monitoring Immune Response in Melanoma". Biomedical Engineering Society (BMES) Annual Meeting, October 7-10, 2015 in Tampa, Florida

7. Participants and Collaborating Organizations

Name	Timothy Bullock, Ph.D.
Project Role	Principal Investigator
Research Identifier	
Nearest Person Month Worked:	2

Contribution to Project	Dr Bullock directed the project's development, guided the assessment of TIL binding, and generated reports
Funding support	NCI
Name	Kimberly Kelly, Ph.D.
Project Role	Co-Investigator
Research Identifier	
Nearest Person Month Worked:	1
Contribution to Project	Dr Kelly mentored Mr Bauknight and supervised the phage display and screening aspects of the project, along with the development of the NGS approaches.
Funding support	NCI;
Name	Dustin Bauknight
Project Role	Graduate Student
Research Identifier	
Nearest Person Month Worked:	12
Contribution to Project	Mr Bauknight has been responsible for the panning of T cell populations and selection of phage; phage sequencing; and in vivo imaging of phage
Funding support	NCI
Name	Andrew Buckner
Project Role	Lab specialist
Research Identifier	
Nearest Person Month Worked:	8
Contribution to Project	Mr Buckner grew tumors; sorted TILs and other T cell populations; and ran the flow cytometry screens and functional assays of T cells incubated with phage.
Funding support	NCI

Change in active other support: Nothing to Report

Other partner organizations: Nothing to Report

8. **Special Reporting Requirements**: none

9. **Appendices**: none